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Hsp70 inhibits lipopolysaccharide-induced NF-κB activation by interacting with TRAF6 and inhibiting its ubiquitination

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Abstract **Inducible heat shock protein 70 (Hsp70) is one of the most important HSPs for maintenance of cell integrity during normal cellular growth as well as pathophysiological conditions. Tumor necrosis factor receptor-associated factor 6 (TRAF6) is a crucial signaling transducer that regulates a diverse array of physiological and pathological processes and is essential for activating NF-κB signaling pathway in response to bacterial lipopolysaccharide (LPS). Here we report a novel mechanism of Hsp70 for preventing LPS-induced NF-κB activation in RAW264.7 macrophage-like cells. Our results show that Hsp70 can associate with TRAF6 physically in the TRAF-C domain and prevent TRAF6 ubiquitination. The stimulation of LPS dissociates the binding of Hsp70 and TRAF6 in a time-dependent manner. Hsp70 inhibits LPS-induced NF-κB signaling cascade activation in heat-shock treated as well as Hsp70 stable transfected RAW264.7 cells and subsequently decreases iNOS and COX-2 expression. Two Hsp70 mutants, Hsp70ΔC(1–428aa) with N-terminal ATPase domain and Hsp70C(428–642aa) with C-terminal domain, lack the ability to influence TRAF6 ubiquitination and TRAF6-triggered NF-κB activation. Taken together, these findings indicate that Hsp70 inhibits LPS-induced NF-κB activation by binding TRAF6 and preventing its ubiquitination, and results in inhibition of inflammatory mediator production, which provides a new insight for analyzing the effects of Hsp70 on LPS-triggered inflammatory signal transduction pathways.**

Keywords: Heat shock protein 70; Tumor necrosis factor receptor-associated factor 6; Lipopolysaccharide; NF-κB; Ubiquitination

1. Introduction

Lipopolysaccharide (LPS), a principal component of gram-negative bacteria, activates variety of the mammalian cell types, including monocytes/macrophages and endothelial cells and contributes to systemic change known as septic shock [1]. In systemic inflammatory response syndrome (SIRS), LPS could induce expression of various pro-inflammatory mediators including TNF-α, IL-1, IL-6, and nitric oxide (NO) in monocyte/macrophages [1–4]. It has been well demonstrated that nuclear factor-κB (NF-κB) and mitogen-activated protein kinases (MAPKs) cascades are two major signaling pathways which mediated LPS-induced inflammatory cytokines production [5–8].

NF-κB, an essential transcriptional factor in the regulation of a variety of genes expression, is involved in immune function, inflammatory responses, and the control of cell growth [9–11]. NF-κB is normally present in the cytoplasm forming an inactive complex with an inhibitor known as IκBα. LPS-elicited NF-κB activation is initiated by the interaction between the Toll-like receptor 4 (TLR4) and myeloid differentiation protein 88 (MyD88), followed by stimulation of IL-1 receptor-associated kinase-1 (IRAK-1) and subsequent recruitment of tumor necrosis factor receptor-associated factor 6 (TRAF6). Stimulation of these proximal signal molecules results in the phosphorylation of IκB kinase α/β (IKKα/β) and IκBα, and concomitant release and activation of NF-κB [12–15].

The heat shock proteins (HSPs) represent a family of proteins that are phylogenetically well conserved and are known to function as molecular chaperones during protein folding, assembly and membrane translocation, and to prevent aggregation of misfolded polypeptide chains in cells. HSPs are present in almost all eukaryotic cells and are transiently over-expressed when cells are exposed to heat-shock [16,17]. Inducible heat shock protein 70 (Hsp70) is the major heat shock protein that is thought to protect cells in a variety of human diseases, including infection, ischemia, and inflammation [18–20]. Hsp70 is actively synthesized in macrophages which are exposed to bacterial toxins, lipid mediators, oxygen free radicals and nitric oxide [21,22]. It has been reported that Hsp70 inhibited LPS-stimulated production of inflammation mediators such as TNF-α, IL-1 and inducible nitric oxide synthase (iNOS) [23,24]. The inhibitory effects of heat-shock on LPS-induced iNOS production in astrocytes, macrophages and other cell types are mainly mediated by expression of Hsp70 [24,25]. In addition, it has been well studied that over-expression of Hsp70 protects rat pancreatic islet β-cells against IL-1β stimulation and blocks TNF-α production in macrophage [26,27]. These findings suggest that induction of

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Abbreviations: Hsp70, heat shock protein 70; TRAF6, tumor necrosis factor receptor-associated factor 6; LPS, lipopolysaccharide; NF-κB, nuclear factor-κB; IκBα, inhibitor of NF-κB α; IKK, IκB kinase; MAPKs, mitogen-activated protein kinases; JNK, c-Jun N-terminal kinase; iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase-2

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Hsp70 may protect the host by regulating LPS-induced production of cytokines in macrophages. However, the mechanisms underlying these protective effects are still unclear.

In the present study, we demonstrate that Hsp70 physically associates with TRAF6 to inhibit LPS-triggered TRAF6 ubiquitination, and subsequently suppresses NF-κB cascade activation. These results show a novel mechanism of Hsp70 for LPS-induced immune response.

2. Materials and methods

2.1. DNA constructs

pcDNA3-HA-Ubiquitin, 2 × NF-κB-Luc reporter vector were generous gifts from Dr. Ze'ev Ronai (The Burnham Institute, USA), pCR-Flag-TRAF6 was gift from Dr. Zhijie Chang (Tsinghua University, PR China). pcDNA3.1-Xpress-Hsp70 and pcDNA3-Flag-Hsp70 were constructed by PCR from human embryonic liver cDNA library using appropriate restrictive enzymes. The HA-tagged TRAF6 and truncated TRAF6 constructs were made by PCR from pCR-Flag-TRAF6 and cloned into pCR vectors, and the Hsp70 truncated constructs were from pcDNA3.1-Xpress-Hsp70. All expression vectors were sequenced to confirm.

2.2. Antibodies and reagents

Polyclonal antibodies against JNK/SAPK, phospho-JNK/SAPK (Thr183/Tyr185), p38 MAPK, phospho-p38 MAPK (Thr180/Tyr182), p42/p44 MAPK, phospho-p42/44 MAPK (Thr202/Tyr204), phospho-IκBα (Ser32) and phospho-IKKα (Ser180)/IKKβ (Ser181) were obtained from Cell Signaling Technology. Antibodies against TRAF6 (H-274), COX-2 (C20) and Ub (P4D1) were from Santa Cruz Biotechnology. Monoclonal antibody against Hsp70 was from Stressgen Bioreagents. Monoclonal antibody against Xpress-tag was purchased from Invitrogen. Monoclonal antibody against Flag-tag was from Sigma. The anti-iNOS monoclonal antibody was purchased from BD Pharmingen. All secondary antibodies used for Western blotting were purchased from Calbiochem.

LPS (from *Escherichia coli* 0111 : B4) was purchased from Sigma. G418 sulfate was from Calbiochem.

2.3. Cell culture and transfection

The murine macrophage-like cell line RAW264.7 and human embryonic kidney cell line HEK293 were cultured in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% (v/v) fetal calf serum (Hyclone) and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) at 37 °C in an atmosphere of 5% CO_2.

Transient transfection was performed with a modified calcium phosphate method or by the LipofectAMINE 2000 reagent (Invitrogen) according to the manufacture's instructions. In all cases, the total amount of DNA was normalized by the empty control plasmids. To generate stable cell line expressing Flag-Hsp70, pcDNA3 vector with or without Flag-tagged Hsp70 sequence was transfected into RAW264.7 cells using LipofectAMINE 2000. Seventy-two hours after transfection, the cells were incubated in fresh medium containing 500 μg/ml G418 for 4 weeks. Subsequently, cell colonies resistant to G418 were isolated and screened by limited dilution. Hsp70 stable expression clones and pcDNA3 control clones of RAW264.7 cells were selected for further studies.

2.4. Luciferase reporter assays

RAW264.7 cells or HEK293 cells cultured in 24-well plates were transiently transfected with NF-κB target sequence linked-luciferase reporter plasmid together with indicated expression vectors. Cell lysates were then prepared and the luciferase activity was measured using Luciferase Assay System (Promega) and analyzed by the Luminometer TD-20/20 (Turner Co. Ltd.). pCMV-β-galactosidase expression vector was added in each transfection and the β-galactosidase assay was carried out as described previously [28] to normalize the transfection efficiency. Each transfection was performed at least twice with triplicates.

2.5. Immunoprecipitation and immunoblotting analysis

Cells were rinsed twice with ice-cold PBS, and solubilized in lysis buffer containing 20 mM Tris (pH 7.5), 135 mM NaCl, 2 mM EDTA, 2 mM DTT, 25 mM β-glycerophosphate, 2 mM sodium pyrophosphate, 10% glycerol, 1% Triton X-100, 1 mM sodium orthovanadate, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride (PMSF) for 30 min on ice. Lysates were centrifugated (15000 × *g*) at 4 °C for 15 min. Proteins (500 μg) were immunoprecipitated for 2 h with indicated antibodies (0.5 μg) respectively. The precleared Protein A/G PLUS-agarose beads (Santa Cruz Biotechnology) were incubated with immunocomplexes for another 2 h and washed four times with the lysis buffer. The samples were separated by SDS–PAGE, transferred to a PVDF membrane, and detected by Western blot analysis. The proteins were visualized using Lumi-Light Western Blotting Substrate (Roche Applied Science) or using TMB immunoblotting system (Promega). The total density of the protein bands was calculated by using of Gel Doc2000 (Bio-Rad). Aliquots of whole cell lysates were subjected to immunoblotting analysis to confirm appropriate expression of proteins.

3. Results

3.1. Hsp70 physically interacts with TRAF6

The inhibitory effects of heat-shock on LPS-induced NF-κB activation and pro-inflammatory mediator production in macrophages and other cell types are mainly mediated by expression of Hsp70 [24,25]. On the other hand, gene disruption experiments showed that TRAF6 is essential for LPS-activated NF-κB signaling pathway [29,30]. These reports suggested that Hsp70 regulate LPS-elicited signaling cascade via directly targeting on TRAF6.

To prove our hypothesis, murine macrophage-like RAW264.7 cells were cultured at 41 °C for 30 min followed by 37 °C for different time. Immunoblotting showed that Hsp70 obviously induced after heat shock, while Hsp90, another important member of HSPs, has no markedly change (Fig. 1A). Co-immunoprecipitation showed that endogenous TRAF6 specifically associated with heat-shock-induced Hsp70, but not Hsp90. Furthermore, the amount of TRAF6–Hsp70 complex decreased to minimum 20 min after LPS treatment, and then returned to the basal level at 60 min, whereas the Hsp70 protein level did not change within this period (Fig. 1B). Recent study showed that Hsp70 could bind to IKKγ and impair TNF-α-induced NF-κB signaling [31]. However, the interaction of Hsp70 and IKKγ was not affected by LPS in the present experiment (Fig. 1B).

We further investigated the interaction of Hsp70 with TRAF6 in HEK293 cells under over-expression conditions. HEK293 cells were co-transfected with Xpress-tagged Hsp70 and Flag-tagged TRAF6 expression vectors or empty vector respectively, then the cell lysates were subjected to immunoprecipitation using anti-Flag antibody. The results of immunoblotting analysis using anti-Xpress antibody revealed that Hsp70 obviously interacted with TRAF6 (Fig. 1C). Taken together, these results demonstrated that Hsp70 physically associated with TRAF6.

3.2. The TRAF-C domain of TRAF6 is necessary for Hsp70 binding

TRAF6 is an adaptor protein with the RING finger and zinc finger motifs for mediating downstream signaling events and the TRAF-C domain for interacting with upstream receptors and other signaling molecules (Fig. 2A). To determine the region of TRAF6 responsible for the binding with Hsp70, differ-

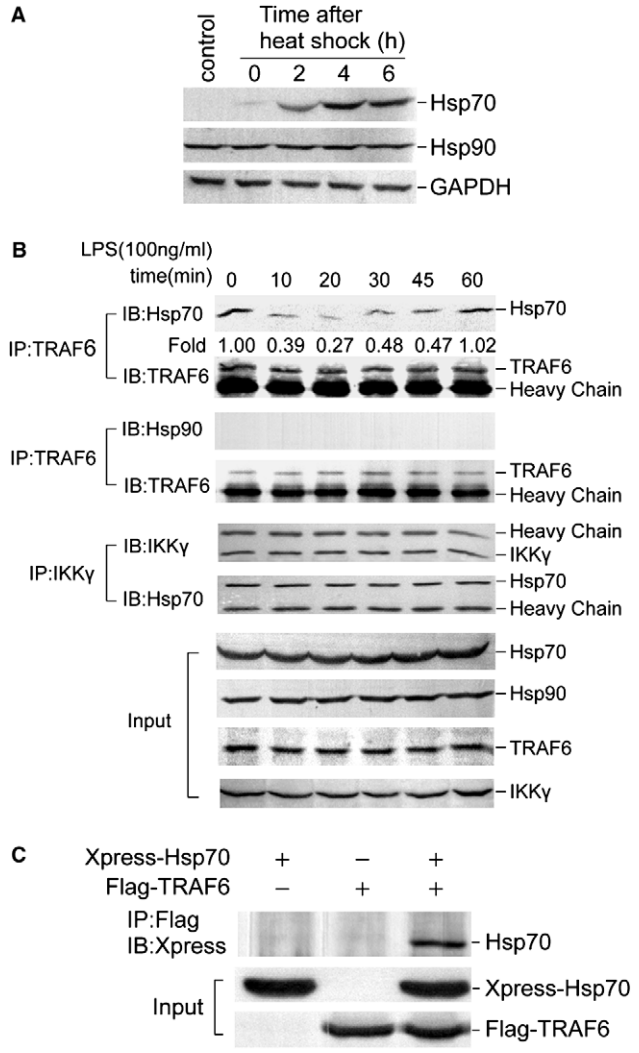

Fig. 1. Hsp70 binds with TRAF6. (A) RAW264.7 cells were either untreated or exposed to a mild heat shock (41 °C for 30 min) and recovered at 37 °C for up to 6 h. The expression level of Hsp70 and Hsp90 were determined by immunoblot analysis. (B) RAW264.7 cells were exposed to a mild heat shock (41 °C for 30 min) and then incubated for 4 h at 37 °C, followed by LPS (100 ng/ml) treatment for indicated time periods. Cell lysates were prepared and subjected to immunoprecipitation with anti-TRAF6 antibody or anti-IKKγ antibody respectively. The immunopellets were analyzed by immunoblot analysis with anti-Hsp70 or anti-Hsp90 antibody. The fold change of Hsp70–TRAF6 complex in LPS stimulated cells compared with untreated cells was determined by densitometer. The amounts of Hsps, TRAF6 and IKKγ protein level in the cell lysates were determined by immunoblotting. (C) HEK293 cells were co-transfected with Flag-Tagged TRAF6 and Xpress-tagged Hsp70 plasmids or empty expression vectors as indicated. 36 h after transfection, the cells were lysed and the lysates were immunoprecipitated with anti-Flag antibody and then analyzed by immunoblotting with anti-Xpress antibody.

ent Flag-tagged TRAF6 truncated mutants were constructed. HEK293 cells were co-transfected with the Flag-tagged TRAF6 mutants and Xpress-tagged Hsp70 respectively. The immunoblotting analysis of co-immunoprecipitation showed that the TRAF-C domain but not the RING finger or zinc finger motif of TRAF6 was required for the interaction of TRAF6 with Hsp70 (Fig. 2B).

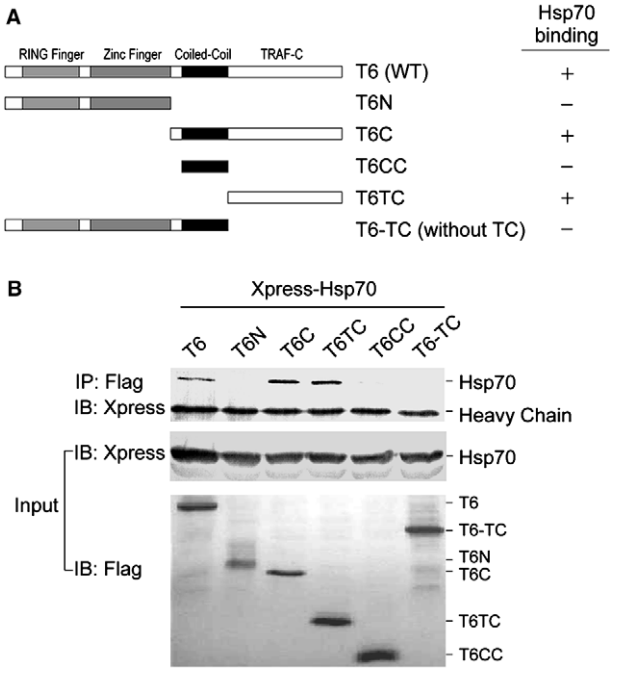

Fig. 2. Mapping of TRAF6 domains required for Hsp70 binding. (A) Schematic diagram of wild type TRAF6 and its deletion mutants. Their abilities to interact with Hsp70 are shown as '+', binding of Hsp70 with the respective TRAF6 region, and as '–', lacking of such interaction in this assay. (B) TRAF-C domain is essential for the binding of TRAF6 with Hsp70. Xpress-tagged Hsp70 was transiently co-transfected with Flag-tagged full length or truncated mutants TRAF6 into HEK293 cells. Thirty-six hours after transfection, cell lysates were subjected to immunoprecipitation with anti-Flag antibody. Co-precipitated Xpress-Hsp70 was detected by immunoblot analysis with anti-Xpress antibody. Expression of Hsp70 and TRAF6 wild type and deletion proteins was determined by immunoblotting respectively.

Hsp70, the member of HSP family, is consisted of N-terminal ATPase domain and C-terminal domain including polypeptide binding motif and variable region (Fig. 3A). To determine the region of Hsp70 responsible for binding with TRAF6, we constructed two Xpress-tagged Hsp70 truncation mutants, Hsp70ΔC (1–428aa) with N-terminal ATPase domain and Hsp70C (428–642aa) containing C-terminal peptide binding domain. HEK293 cells were co-transfected with HA-tagged TRAF6 and Xpress-tagged Hsp70ΔC or Hsp70C, followed by co-immunoprecipitation using anti-Xpress antibody and Western Blotting with anti-HA antibody. The data revealed that the C-terminal but not the N-terminal of Hsp70 was required for the specific association of Hsp70 with TRAF6 (Fig. 3B).

3.3. Hsp70 inhibits LPS- and TRAF6-induced NF-κB activation

Above data indicated that Hsp70 could bind to TRAF6 and play a regulatory role in LPS-activated signaling cascades. Thus the role of Hsp70 in regulating LPS/TRAF6-activated NF-κB was first addressed in transfection studies using NF-κB-luciferase reporters. RAW264.7 cells were co-transfected with reporter construct and Xpress-tagged Hsp70 expression vector as indicated. 24 h after transfection, the cells were treated with LPS (500 ng/ml) for 12 h, and then the luciferase activities were assayed. The NF-κB Luciferase activity level in Hsp70 transfected RAW264.7 cells was lower than that in

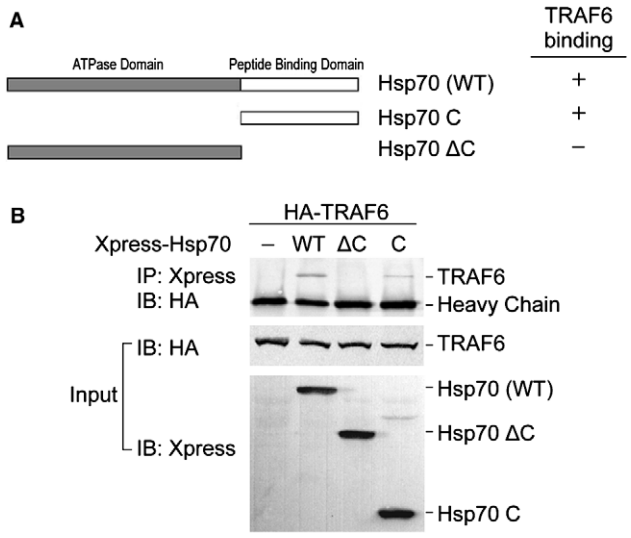

Fig. 3. C-terminal of Hsp70 is required for TRAF6 binding. (A) Schematic diagram of full length Hsp70 and its two structural mutants. Their abilities to interact with TRAF6 are shown as '+', binding with TRAF6, and as '−', lacking of such binding. (B) Lysates from HEK293 cells transfected with HA-tagged TRAF6 and Xpress-tagged full length or truncated Hsp70 were subjected to immunoprecipitation with anti Xpress antibody followed by immunoblotting with anti HA antibody (upper panel). Lysates (input) were immunoblotted with anti HA and anti Xpress antibodies to determine the expression of TRAF6 and Hsp70 (lower panels).

control cells after LPS treatment (Fig. 4A). To determine the role of Hsp70 in regulating TRAF6-mediated NF-κB activation, HEK293 cells were co-transfected with NF-κB reporters together with Flag-tagged TRAF6 or empty vector respectively, and the cell lysates were subjected for luciferase activities assay 36 h after transfection. Similar as above result, Hsp70 also obviously suppressed TRAF6-mediated NF-κB activation (Fig. 4B).

To further investigate the physiological effect of Hsp70 on regulating LPS-stimulated NF-κB signal pathway, we established stable Hsp70-expressing cells by transfection of Flag-Hsp70 cDNA into RAW264.7 cells and subsequent selection of stable transfected clones using G418. The expression of Hsp70 in one clone used for the majority in the experiments was as similar as that of heat-shock-induced Hsp70 in normal RAW264.7 cells and at the physiological level. As shown in Fig. 4C, Hsp70 apparently inhibited LPS-induced IKKα/β phosphorylation and IκBα degradation, which was consistent with that of heat shock-treated RAW264.7 cells (Fig. 4C). However, LPS-stimulated activation of extracellular signal-regulated kinase, c-Jun N-terminal kinase (JNK) and p38 was similar in the stable Hsp70-expressing and control RAW264.7 cells (Fig. 4D). In addition, the result from immunoprecipitation using anti-Hsp70 antibody followed by immunoblotting analysis using anti-IKKα or IKKβ antibody did not show any Hsp70-IKKα/β complex (Fig. 5A), which is coincidence with the finding that Hsp70 and Hsc70 did not associate with IKK complex [32]. These data suggest that Hsp70 inhibits LPS-stimulated NF-κB activation via blocking phosphorylation of IKK.

In order to determine if IKK-induced activation of NF-κB can be regulated by Hsp70, HEK293 cells were co-transfected with NF-κB reporters together with IKKβ and Hsp70 or

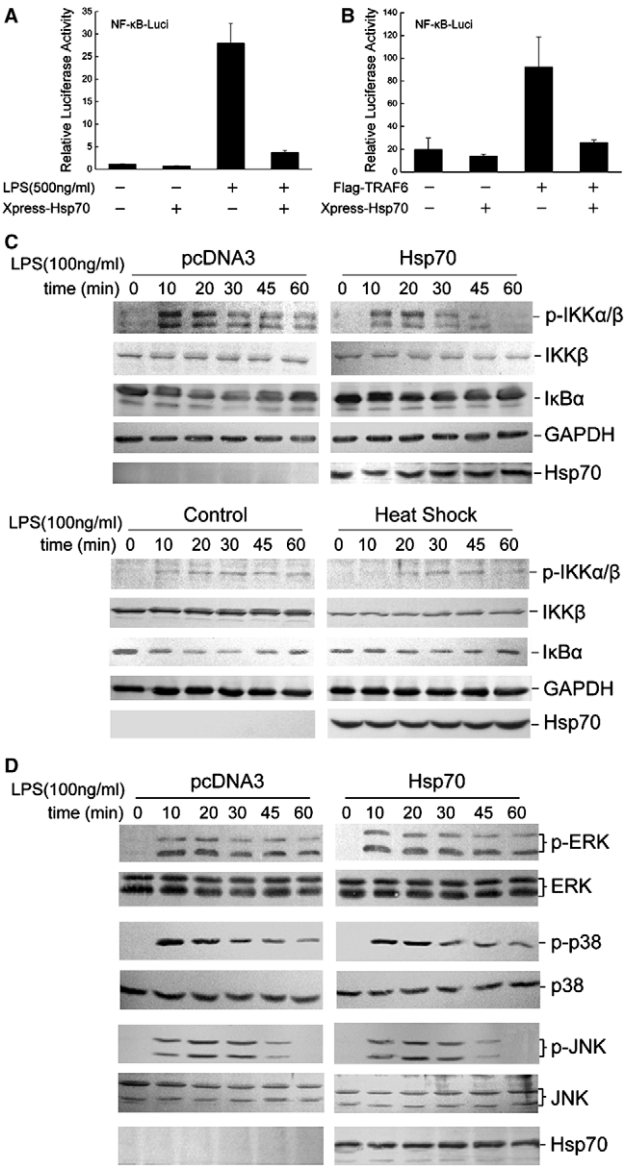

Fig. 4. Hsp70 suppresses NF-κB signaling cascade. (A) RAW264.7 cells cultured in 24-well plates were transiently transfected with Xpress-tagged Hsp70 and NF-κB luciferase reporter plasmid. Twenty-four hours after transfection, cells were treated with or without LPS (500 ng/ml) for 12 h, and then the luciferase activities were measured using a luciferase assay system. (B) HEK293 cells were co-transfected with Flag-tagged TRAF6 and Xpress-tagged Hsp70 along with NF-κB reporter. Luciferase activity was measured 36 h after transfection. All values were normalized based on β-gal activities. Values shown are averages (mean ± SD) of one representative experiment in which each transfection was performed in triplicate. (C) Stable pcDNA3 and Hsp70-expressing RAW264.7 clones (upper panels), and normal RAW264.7 cells treated by heat shock or not (lower panels) were incubated with 100 ng/ml LPS for indicated time course. IκBα, IKKβ and phosphorylated IKKα/β were determined by immunoblotting with specific antibodies. The levels of heat-shock-induced and forced-expressed Hsp70 were determined by immunoblotting with anti-Hsp70 antibody. (D) Stable Hsp70-expressing and control RAW264.7 cells were treated with LPS (100 ng/ml) for different time intervals. The phospho-MAPKs and total MAPKs in cell lysates were detected by immunoblotting respectively.

empty vector respectively, and the cell lysates were subjected to luciferase activities assay 36 h after transfection. Being consistent with the previous reports that Hsp70 can maintain IκB

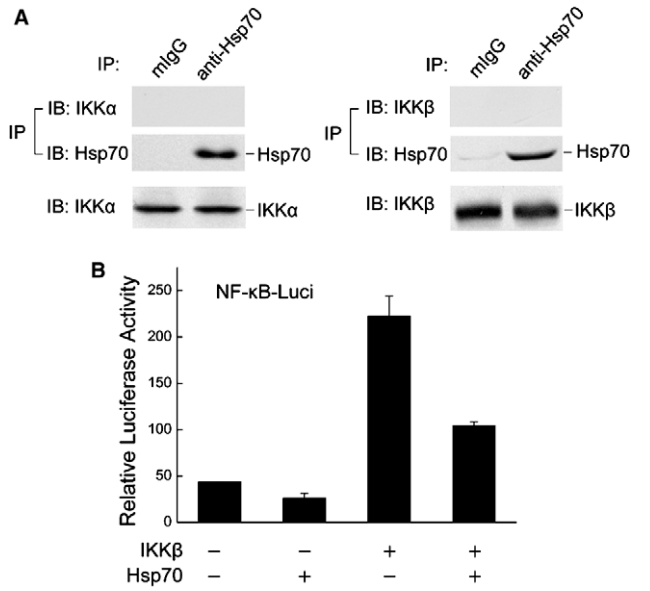

Fig. 5. Hsp70 does not co-precipitate with IKKα or IKKβ. (A) Lysates from heat shock-treated RAW264.7 cells were subjected to immunoprecipitation with anti Hsp70 antibody or control mouse IgG followed by immunoblotting with anti IKKα (left panels) or IKKβ (right panels) antibody. Lysates were immunoblotted with specific antibodies to detect the existence of IKKα and IKKβ proteins. (B) HEK293 cells were co-transfected with IKKβ and Xpress-tagged Hsp70 along with NF-κB reporter. Luciferase activity was measured 36 h after transfection. All values were normalized based on β-gal activities. Values shown are averages (mean ± SD) of one representative experiment in which each transfection was performed in triplicate.

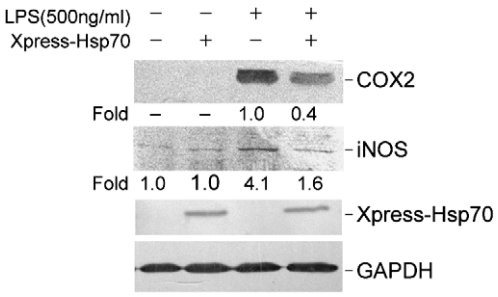

Fig. 6. Hsp70 suppresses LPS-induced COX2 and iNOS expression. RAW264.7 cells were transfected with Xpress-tagged Hsp70. Twenty-four hours after transfection, the cells were exposed to LPS (500 ng/ml) for 10 h, and the cell lysates were prepared. COX2 and iNOS expression levels were determined by immunoblotting using anti-COX2 antibody and anti-iNOS antibody respectively. The fold decrease in COX2 and iNOS protein level in Xpress-Hsp70-expressing RAW264.7 cells compared with control cells was determined by densitometer (upper 2 panels). Equal loading protein was confirmed by immunoblotting with anti-GAPDH antibody.

stability through protein–protein interaction, over expression of Hsp70 indeed blocked IKK-mediated activation of NF-κB (Fig. 5B) in the present experiment [33].

3.4. Hsp70 inhibits LPS-induced iNOS and COX-2 production

NO and prostaglandins, produced by iNOS and cyclooxygenase-2 (COX-2) respectively, are two kinds of important mediators of inflammatory and immune responses induced by various stimuli including LPS and cytokines. iNOS and COX-2 expression are largely controlled by NF-κB signaling pathway upon LPS stimulation [25,34]. Therefore we next determined the effect of Hsp70 on iNOS and COX-2 expression in LPS-stimulated RAW264.7 cells. The data showed that overexpression of Hsp70 significantly inhibited LPS-induced production of both iNOS and COX-2 compared with control cells (Fig. 6), suggesting that Hsp70, through interacting with TRAF6, inhibits NF-κB mediated inflammatory factor production in response to LPS.

3.5. Hsp70 suppresses TRAF6 ubiquitination

In TLR4-mediated signaling, TRAF6 was recruited into the complex via its association with IRAK-1, and then the dimerization of TRAF6 triggered a K63 poly-Ub chain conjunction on itself. The ubquitination of TRAF6 mediates the activation of NF-κB through proteasome-dependent pathway, but does not induce TRAF6 itself degradation [35,36]. We further studied if the binding of Hsp70 with TRAF6 could affect the auto-ubiquitination of TRAF6. HEK293 cells were co-transfected with HA-tagged ubiquitin, Flag-tagged TRAF6 and Xpress-tagged Hsp70 expression vectors including wild Hsp70, Hsp70ΔC or Hsp70C as indicated. The immunoprecipitation was performed with anti-Flag antibody and probed with the antibody against HA to assess the degree of TRAF6 ubiquitination. As shown in Fig. 7A, lower ubiquitinated TRAF6 level was observed in Hsp70-overexpressing cells, suggesting that Hsp70 could suppress TRAF6 ubiquitination. However, the two Hsp70 truncated mutants had no effect on that (Fig. 7C). This result is consistent with the finding that intact Hsp70 is necessary for the inhibition of TRAF6-mediated NF-κB activation (Fig. 7D). To evaluate the effects of Hsp70 on endogenous TRAF6 ubiquitination, the levels of ubiquitinated TRAF6 were detected in LPS-treated stable Hsp70-expressing and control RAW264.7 cells. The results revealed that over-expression of HSP70 obviously reduced LPS-stimulated endogenous TRAF6 ubiquitination (Fig. 7B).

Taken together, these data suggested a novel mechanism that Hsp70 regulate LPS-induced NF-κB activation through binding with TRAF6 and preventing TRAF6 ubiquitination.

4. Discussion

NF-κB activation induced by various stimuli is mediated by members of the TRAF adapter family [37,38]. While TRAF2 and TRAF5 play a crucial role in NF-κB activation by TNF-α [39], TRAF6 is essential for activation of NF-κB, JNK and p38 in both LPS and IL-1 signaling [29,30]. Previous studies have shown that Hsp70 and heat shock modulate NF-κB activation, but the mechanism by which Hsp70 inhibits NF-κB activation remained unclear. IKK, composed of two catalytic subunits including IKKα and IKKβ, is able to phosphorylate IκBα and cause its rapid degradation by the ubiquitin (Ub)-proteasome pathway and thereby mediates NF-κB activation. IKKγ is an essential regulatory component of the IKK complex that is necessary for NF-κB activation. Recently, it was reported that the ability of Hsp70 to promote TNF-mediated apoptosis was attributed to binding with IKKγ and impairing NF-κB survival signaling [31]. In the present study, we found that heat shock-induced Hsp70 associates with TRAF6 and IKKγ in unstimulated RAW264.7 cells. However, LPS only mediated Hsp70 dissociation from TRAF6

but did not affected Hsp70-IKKγ binding (Fig. 1), furthermore, Hsp70 suppressed LPS- and TRAF6-induced NF-κB activation. These results indicated that Hsp70 may inhibit NF-κB activation by protein–protein interacting with TRAF6. Beside as a signal transducer in the NF-κB pathway and IKK activation, TRAF6 is also required for TAK1-mediated phosphorylation of MKK6 in the JNK-p38 pathway [40]. By comparing the effect of Hsp70 on IKKα/β, IκBα and MAPKs signal pathway, we found that Hsp70 inhibited LPS-induced IKKα/β phosphorylation and IκBα degradation, but not MAPKs activation (Fig. 4). Hsp70 also inhibited LPS-triggered and NF-κB-mediated iNOS and COX-2 production (Fig. 6).

Upon ligands stimulation, TRAF6 is recruited into the signaling complex via its association with IRAK1 for mediating downstream signaling [36]. Ubiquitination, which involves the covalent attachment of several subunits of ubiquitin to a given substrate via lysine residues, plays an important role in regulating immune and inflammatory responses. TRAF6 is an ubiquitin ligase that is activated by ubiquitination and a signaling intermediate between IRAK1 and IκBα. Different from degradation dependent on proteasome system, ubiquitination of TRAF6 is important for the phosphorylation of IKK [35]. Our study indicates that Hsp70 suppresses TRAF6 ubiquitination via protein–protein interaction with TRAF6, which provides a novel mechanism by which Hsp70 inhibits LPS induced NF-κB activation.

The carboxy-terminal is highly conserved in TRAF family proteins and contains coiled-coil domain and TRAF-C domain [38]. In TRAF6 molecule, the TRAF-C domain serves as an adaptor module which is responsible for the TRAF6 and IRAK1 binding and TRAF6 oligermerization, thus it is critical for TRAF6 to transduce upstream signal upon stimuli and activate downstream kinase IKK [40]. Through analyzing the interaction of Hsp70 with TRAF6 or TRAF6 truncations in HEK293 cells under over-expression condition, we identified that the TRAF-C domain is critical for Hsp70 binding (Fig. 2B). It is probably that heat shock treatment or Hsp70 overexpression result in Hsp70 binding with TRAF6 in the TRAF-C domain. LPS stimulation can disrupt this binding, and thereby make TRAF6 partly release from the complex to transduce the NF-κB signaling.

TRAF6 is required for the activation of both IKK and JNK, but our study showed that Hsp70 only suppressed LPS-induced IKK phosphorylation while it has no effect on LPS-activated JNK. This result suggests that TRAF6 is not the only factor required for transducing LPS signaling to JNK, and Hsp70 specifically downregulates NF-κB signal pathway after LPS stimulation by directly binding with TRAF6.

Heat shock was found to suppress NF-κB-dependent pro-inflammatory cytokine production, and thus was attributed

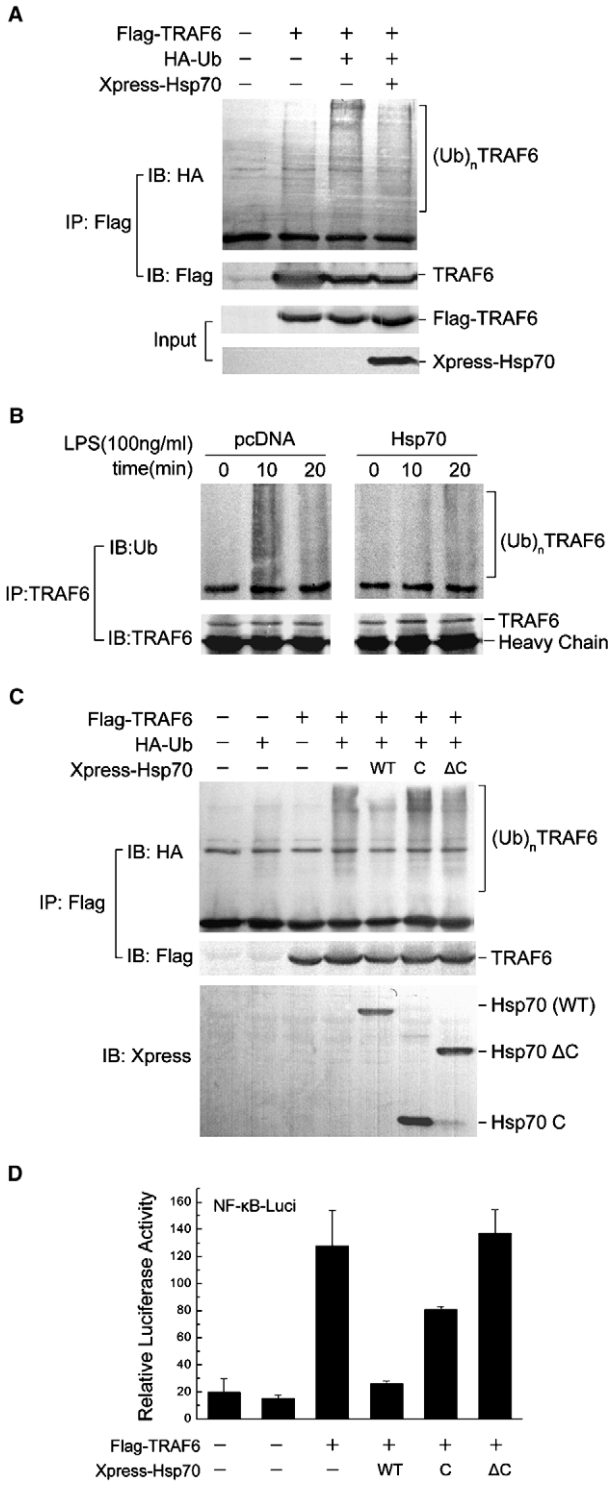

Fig. 7. Full length Hsp70 inhibits ubiquitination of TRAF6 and blocks the activation of NF-κB. (A) HEK293 cells were co-transfected with Flag-tagged TRAF6, HA-tagged Ub or Xpress-tagged Hsp70 constructs. Forty hours after transfection, cell lysates were subjected to immunoprecipitation with anti-Flag antibody and analyzed by immunoblotting with anti-HA antibody or anti-Flag antibody. Over-expressed exogenous proteins in the same lysates were verified by immunoblotting. (B) Stable Hsp70-expressing RAW264.7 cells and control cells were treated with LPS (100 ng/ml) for different time intervals. Cell lysates were subjected to immunoprecipitation with anti-TRAF6 antibody and analyzed by immunoblotting with anti-Ub or anti-TRAF6 antibody. (C) Flag-tagged TRAF6, HA-tagged Ub with various Xpress-tagged Hsp70 constructs were co-transfected into HEK293 cells. Lysates from the HEK293 cells were subjected to immunoprecipitation with anti Flag antibody followed by immunoblotting with anti HA antibody. Lysates (input) were immunoblotted with anti Xpress antibody. (D) Flag-tagged TRAF6 and various Xpress-tagged Hsp70 constructs were co-transfected with NF-κB reporter into HEK293 cells. Luciferase activity was measured 36 h after transfection. All values were normalized based on β-gal activities. Values shown are averages (mean ± SD) of one representative experiment in which each transfection was performed in triplicate.

to blocking cytokine-induced IκBα phosphorylation by inhibiting IKK [41–43]. Whereas, similar study from other investigators showed that accumulated Hsp70 during heat shock negatively regulates the heat-shock-induced suppression of the IκBα/NF-κB pathway [44], and heat shock inhibits activation of the IκBα/NF-κB pathway and NF-κB dependent gene expression in the absence of an intact heat shock response [45]. Our observation demonstrated that IKKβ protein level was obvious decreased in RAW264.7 cells after heat shock, suggesting that heat shock indeed can result in downregulation of IKKβ protein level through their insolubilization, but the recovery of IKKβ protein level was not found in a certain period of time after heat shock even though Hsp70 was induced during this time period. Though Hsp70 is a molecular chaperone, which can bind to some proteins and prevent their insolubilization, we here did not observe that Hsp70 could directly bind to IKKα/β by immunoprecipitation analysis. The mechanism by which heat shock inhibits activation of IκBα/NF-κB pathway in the absence of an intact heat shock response may be contributed by the insolubilization of key kinase such as IKKβ protein after heat shock. Therefore, the differences between the roles of Hsp70 in different study may depend on different type of cells and cellular stimulus used in each study.

In conclusion, our result elucidates a novel function that Hsp70 interacts with TRAF6 by protein–protein binding to inhibit ubiquitination of TRAF6 and provides a new insight for analyzing the mechanism utilized by Hsp70 to prevent injury stimuli, such as LPS, induced NF-κB activation and inflammatory factor production.

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